Exocytosis judged by localized endocytosis after K+ depolarization of chick embryonic peripheral neurons

Whitney Sirois
wsirois@wheatonma.edu
508.286.5201

Abstract

Concentrated K+ in an isosmotic medium can potentially instigate action potentials with in vitro neurons at en passant synapse interactions. Using isolated embryonic chick neurons, we applied isosmotic KCl solution in the presence of rhodamine dextran to track and image localized endocytosis as a result of exocytosis from the generation of an action potential using fluorescent light. The control neurons were administered only rhoadamine dextran solution in HBSS and water. We found that the presence of a K+ isosmotic medium did not create a localized region of endocytosis as a result of exocytosis at en passant axonal regions. No significant change was observed in general axonal regions as a result of K+ and rhodamine dextran administration. From this, we can conclude that the increased concentration of K+ in the extracellular fluid does not stimulate action potentials, that there are no synaptic interactions at en passant axonal regions, the neurons only undergo kiss and run endocytosis. Calcium ionophore administration is recommended for future studies. We can conclude that the KCl solution and rhodamine dextran at these concentrations, though ineffective in these settings, are not harmful to chick embryonic peripheral neurons.

Introduction

Neurons have the ability to communicate with other neurons across a small gap through a process of chemical messaging using small molecules called neurotransmitters. These tiny molecules are distributed from the pre-synaptic cell to the post-synaptic cell through activation of Ca++ channels at the nerve terminal, a result of an action potential in the neuron.

An action potential is a signal in the form of a charge passed down an axon, caused by depolarization of an excitable cell, like a neuron, to the point of elevated (more positive) membrane potential called threshold. At threshold, Na+ channels open due to voltage-sensitive alpha helices in the channel changing their position in the Na+ channel located in the plasma membrane. This movement opens the channel, allowing a rapid influx of Na+ ions, quickly depolarizing the cell. Once the membrane potential reaches a maximum point of Na+ concentration, the electrical and concentration gradients will cause the voltage-gated K+ channels to open while the Na+ channels are deactivated, causing an efflux of K+. This flux decreases the voltage of the membrane, but hyperpolarizes the membrane with an overshoot of K+ ions. The cell then will use Na+/K+ pumps to return the membrane to resting potential. While the cell is returning to normal resting potential, it undergoes a refractory period, in which an action potential cannot occur. This is caused by the K+ influx overshoot (Kandel, 2000).

The release of neurotransmitters is due to synaptic vesicle transport throughout the en passant or nerve terminal region. Synaptic vesicle proteins act to transport the vesicle to the nerve terminal, load the neurotransmitter into the vesicle, associate and dock the vesicle to prepare for release (Harris, et al., 2001). There are three types of endocytosis in a nerve terminal. The classic pathway of endocytosis is the retrieval of extra membrane by clathrin-coated pits (Kandel, 2000). Clathrin is a protein located in the plasma membrane that is recruited to the site of endocytosis. This method is used to recover synaptic proteins and plasma membrane, forming a mature synaptic vesicle. There are three steps in this stage of recycling the vesicle; marking the region of the membrane to be internalized, gathering vesicle proteins to the site to be endocytosed, and finally recruiting clathrin for endocytosis. (Harris, et al., 2001) Another theory endocytosis is the kiss-and-run theory. This mechanism releases the contents of a vesicle through a fusion pore, avoiding complete integration of the vesicle to the plasma membrane. This occurs less often then the classic clathrin-mediated recycling. (Kandel, 2000) Kiss and run prevents some visible tracking techniques, like immersion in rhodamine dextran, from showing the presence of endocytosis. Aprroximately 20% of exo- and endocytosis occurs by this method (Stevens & Williams, 2000). Lastly, the bulk endocytic pathway is recycling excess membrane through uncoated pits. This method of endocytosis is not typically used during normal synapse function, but following a stage of very high rates of release (Kandel, 2000).

In living organisms, the concentration of K+ in the extracellular space is much lower than that of the cytoplasm.

Also in vivo, it has been shown that en passant regions of axons communicate with one another through this process of exocytosis and endocytosis. To test in vitro neurons, dorsal root ganglia and sympathetic nerve chains of chick embryos can be used to plate peripheral neurons in order to investigate the ability to induce action potentials in vitro. Using rhodamine dextran, the presence of endocytosis can be tracked using fluorescent light imaging and phase imaging. This polysaccharide dye used is rhodamine molecule binded to a dextran, which contains a bond that is unbreakable by many organic materials. As a result, dextran will remain in a living and functioning organism for a period of time, allowing dextran to act as a long-term tracer, prime for our imaging techniques. The rhodamine dextran can be added to HBSS medium and water, then viewed with fluorescence microscopy. During this process, green fluorescent light is exposed to the cells at a specific wavelength. The dye, which should occupy the lumen of the cells is absorbs the light and reflects as a red glow. Filters in the microscope detect the light at these wavelengths and make them visible to the human eye. These fluorescent images can be obtained by phase optics to locate regions of en passant axons and simple regions of axons (Cooper, 2004).

In order to reach threshold in these cells, the membrane must be depolarized. At resting potential, a neuron has many K+ channels open, therefore using an excess of K+ in the medium should depolarize the membrane hypothetically to the point of threshold. (Barron, 2004). This experiement will investigate the possibility of determining high amounts of exocytosis, as judged by increased endocytosis recovering extracellular rhodamine dextran in localized small regions of en passant axons when we trigger action potential formation with increased K+ isosmotic medium.

Materials and Methods

Materials:

9-day-old Isolated Chick Neurons (4x density)
Isotonic K+ Buffer: 50mM K+ in 10mL HankÕs Balanced Salt Solution (HBSS) & water (Savage, et. al, 1989)
Rhodamine Dextran: 50mM in K+ Buffer in 4mL HBSS (Bi et al., 1997)
3.5 ml F-medium
Nikon Optiphot-2 microscope
SPOT 2E digital camera
SPOT 2E digital imaging program
Power PC G4 Macintosh Computer
3 Cover slips (22mm X 22mm)
Slide (75mm X 25mm)
Kim wipes
Valap (melted)
Incubator at 37°C
2 Petri dishes (35mm)
Forceps
Sterile pipettes and bulbs

Methods

Chick Peripheral Neuron Preparation

Dorsal root ganglia (DRG) and sympathetic nerve chains were dissected from 8-day-old chick embryos, transferred into small petri dishes, placed on treated coverslips, and stored at 37°C overnight in F-medium. (Hollenbeck & Morris, 1993)

Potassium & HBSS Buffer Preparation

37.3mg of Potassium chloride (KCl) was on an analytical scale. Then, 5.2 ml HBSS was added to KCl in test tube with mechanical pipette for accuracy. With a new pipette, 4.8 ml of distilled water was added into the same test tube, for a 10 ml total. (Savage et al., 1989)

Preparation of Flow Chambers

After viewing plates to be sure of axonal abundance, a cover slip was shattered into chips and arranged 1cm X 1cm in the center of a slide. Then, enough F-medium was dotted to fill chamber in the center of the chards. The F-medium that the cells were growing in was removed with a glass pipette, leaving a thin layer to barely cover cells and allow removal of cover slip to prevent a vacuum. The cover slip was then removed with forceps and placed on chards of glass on the slide. These 9-day-old chick neurons at 4X density, now in a flow chamber were then placed into the incubator at 37°C. (Hollenbeck & Morris, 1993)

Rhodamine Dextran in K+ Buffer & Rhodamine Dextran Control Buffer Preparation

Rhodamine dextran (RD) solution was prepared at a 1:60 dilution of RD in water using 3.3mM, making a 50mM concentration (Bi et al., 1997) in our isosmotic K+ buffer and 50mM in HBSS and water (Savage, et. al). Also, rhodamine dextran in HBSS was prepared, as a medium to use for our control.

Imaging Control Neurons

Using one flow chamber, 0.5 ml of RD control buffer (HBSS and water) was washed through the flow chamber. A kimwipe was used to pull the medium through the other side. After 60 seconds this chamber was washed three times with F-medium, and then the cells were viewed on the Nikon Optiphot-2 microscope using the SPOT 2E digital camera and the SPOT 2E digital imaging program. En passant is defined as regions of axons that interact laterally with no visible distance between the two using phase optics. After finding an area of interest these neurons were imaged using phase optics at 40X and captured, and then under fluorescent settings on the SPOT 2E program, with green light emitted under the microscope, the cells were quickly imaged again (to avoid bleaching). The cells were washed twice more, then imaged on both phase optics and fluorescence in areas of interest.

Imaging Neurons after K+ Induction

Next, the other flow chamber was flushed with RD in the K+ buffer. This was also given 60 seconds, and then washed three times with F-medium, to be imaged on both phase and fluorescence settings. Once again, the cells were washed twice more, then imaged on both phase optics and fluorescence at areas of en passant axons.

Quantification of Data

Using Adobe Photoshop software on a PowerPC G4 Macintosh computer, we circled the tiny areas we considered en passant (see *Imaging Control Neurons*) in the fluorescent pictures. The regions drawn were chosen and classified by the axonal interaction, defined as a region of uniform width of each axon. This was done for both the control and the K+ depolarized cells and then in each fluorescence image, took an additional circled region in a nearby area to define the background or "blackness" of the photo. This region possesses no neuronal cell parts, and a histogram was created for both regions. This provided us with a count of pixels in the given region and the mean luminosity of the outlined image. The mean luminosity of the background was subtracted from the mean luminosity of the synaptic region. From this, we obtain an average brightness of the en passant axonal region of the cell. This method was used for all regions defined as en passant in the images obtained.

This process was repeated on a different part of the axon. An average of the control images was taken, as well as an average of images post K+ administration.

Results

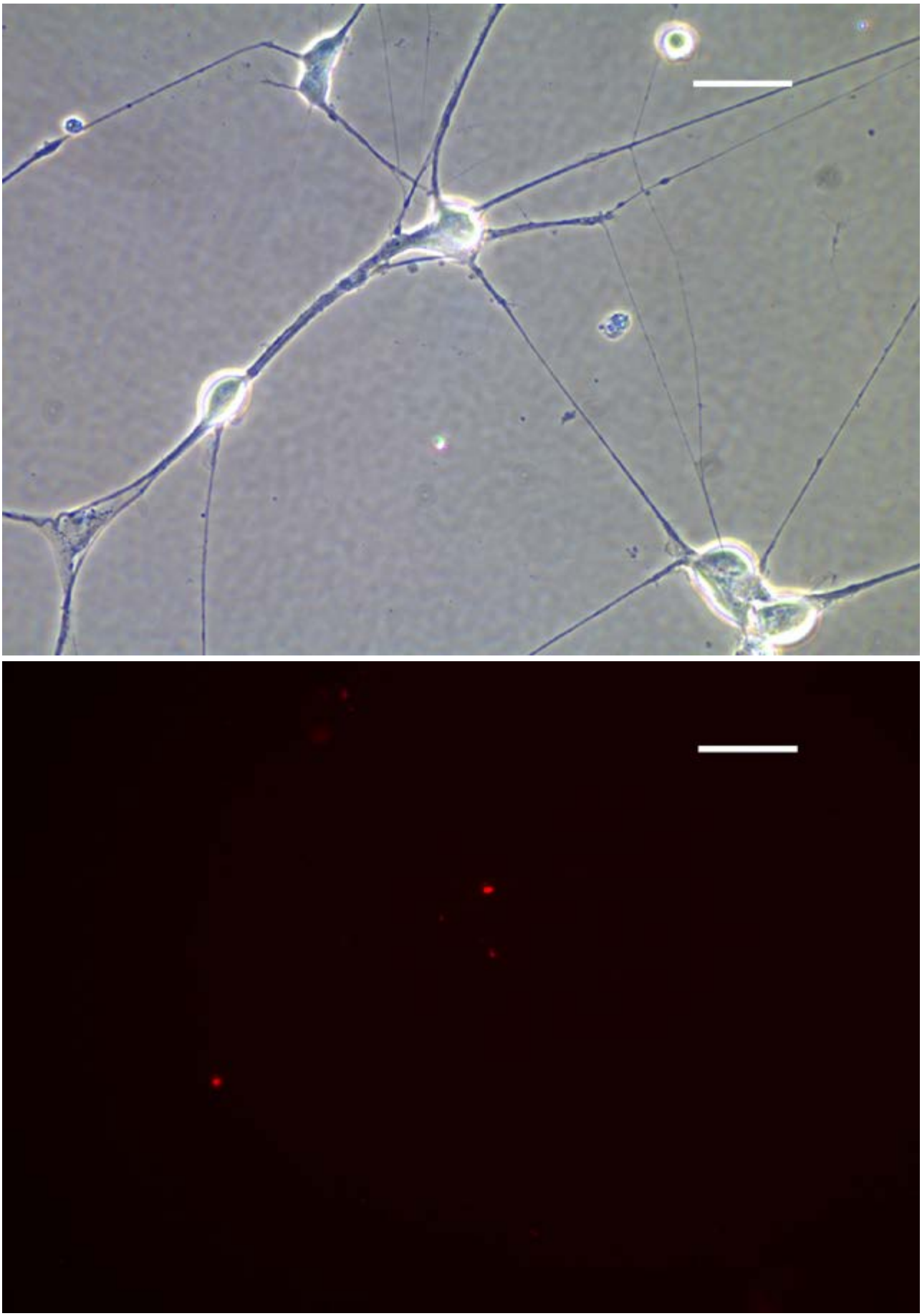

Fig. 1 is an image on phase optics (top) vs. a fluorescent image (bottom) after the control procedure (post rhodamine dextran).

Scale Bar = 50µm

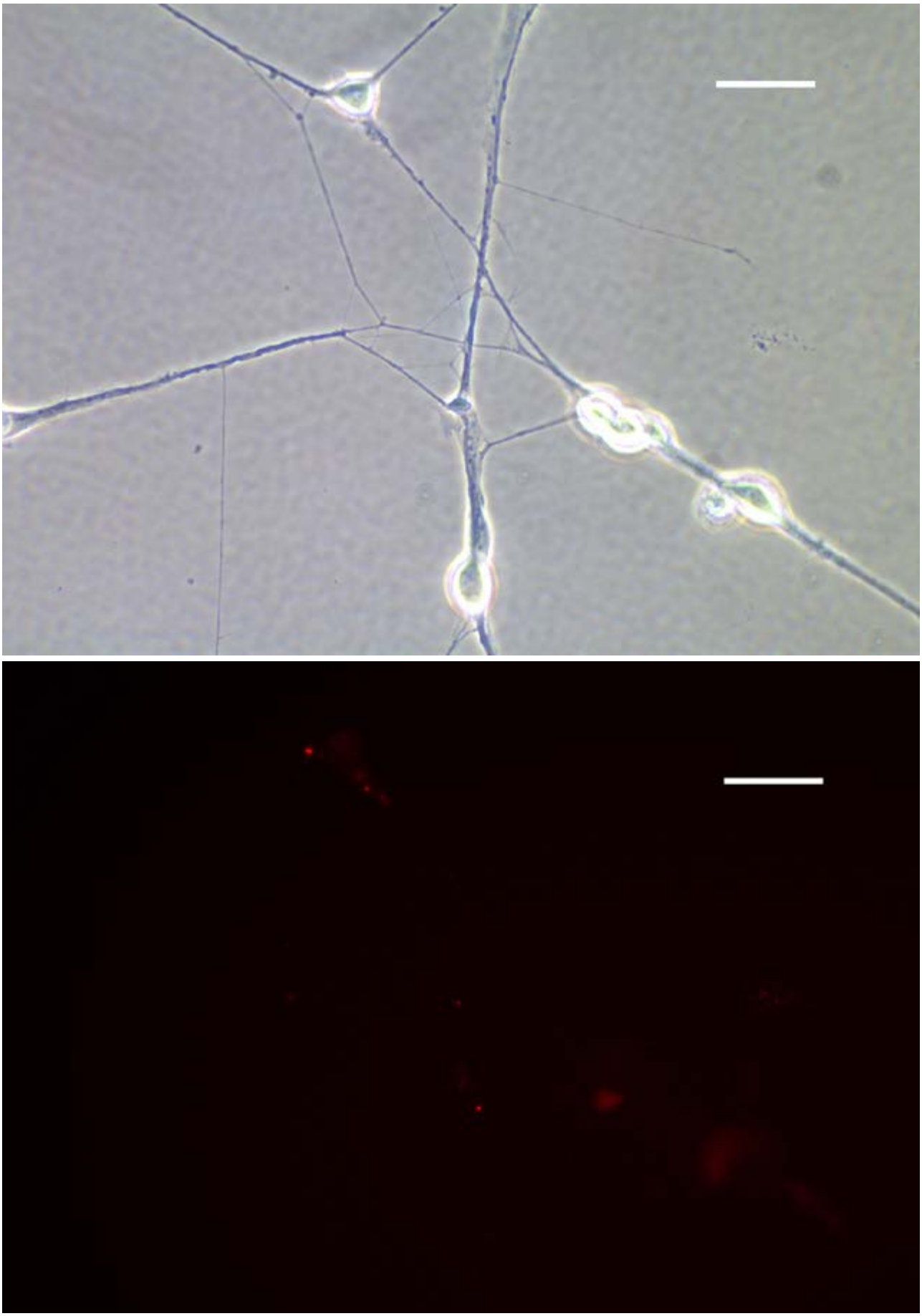

Fig. 2 is an image on phase optics (top) vs. fluorescence image (bottom) after the experimental procedure (post K+

depolarization and post rhodamine dextran).
Scale bar = 50µm

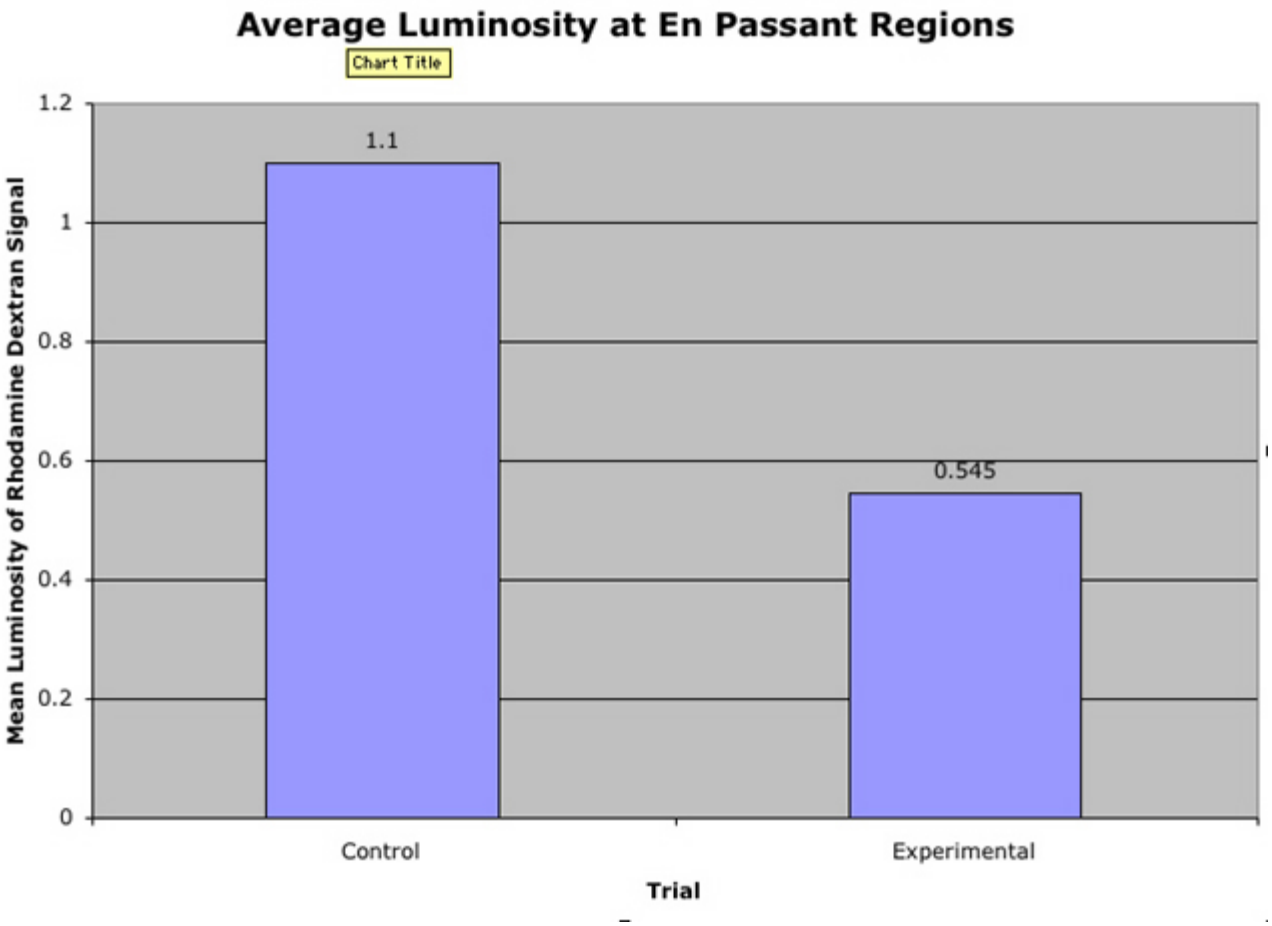

Fig. 3 shows the differences in luminosities in the control and K+ depolarized trials at en passant axons after rhodamine dextran administration.

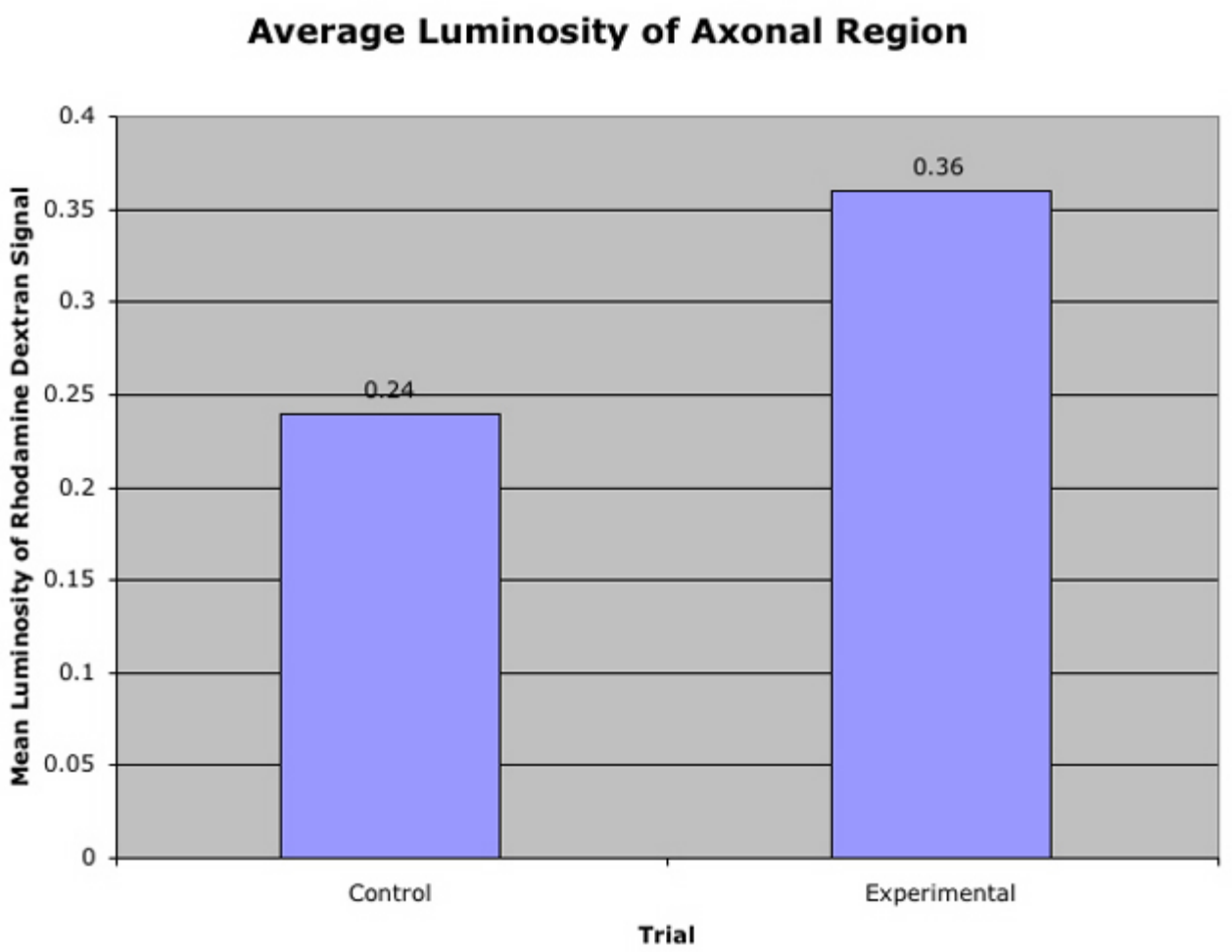

Fig. 4 shows the differences in luminosities in the control and K+ depolarized trials of axonal regions after rhodamine dextran administration.

Discussion and Conclusions

From the data obtained in this experiment, it would be possible to conclude that endocytosis was not observed in these cells under these conditions. Fig. 1 is the control trial of the en passant axons, and the regions of en passant axons do not show brightness, which we expected. Fig. 2 is an image of a phase and a fluorescent image after K+ administration. However, the brightness for the en passant axonal regions in this image doesn't show any significant increase in brightness for the experimental trial. This is also reflected in Fig. 3, the graph of average luminosity at en passant regions. It showed that, in opposition to the original hypothesis, the experimental images showed a smaller difference in mean brightness after removing the background luminosity. The control actually had a larger difference in mean brightness when averaged together.

Then, brightness in axonal regions of the neuron was completed. As you can see in Fig. 4, the results were opposite, but the differences in brightness as compared with the background are minimal. These values are too low and too close to conclude that there would be a significant difference in brightness with and without K+ administration.

It has been shown that en passant synapses in vivo can have synaptic interaction by Maravall and colleagues (2004), but it has not yet been proven that this interaction is possible in vitro. The hypothesis of this experiment is strictly limited to the theory that neurons could synapse at en passant regions in vitro. Perhaps in vitro cells under these conditions cannot produce en passant synaptic interactions.

It is also likely that these peripheral neurons may not have undergone action potentials due to the presence of K+ in the medium. It is possible that the K+ in the medium wasnÕt concentrated enough to depolarize the membrane to threshold, causing the action potential necessary for neurotransmission, therefore causing an endocytosis of the rhodamine dextran into the synaptic terminal.

Another reason that there may have not been a notable occurrence of endocytosis in the cell could be due to the

nature of the endocytosis. Perhaps these chick embryonic peripheral neurons only undergo kiss-and-run endocytosis when exposed to a higher K+ concentration and possible an action potential. Kiss-and-run endocytosis doesn't allow for the tracking by rhodamine dextran because it exists in the medium (the extracellular space), and none of that material would enter a kiss-and-run endocytic neuron.

We are able to conclude, however, that the K+ medium was, in fact, isosmotic. Our cells maintained their shape and function with the addition of KCl and rhodamine dextran to the HBSS and water solutions. The concentrations used are safe for living cells.

For future experiments, it would be useful to use the calcium ionophore A23-187 to track the localization of endocytosis using in vitro neurons. Calcium influx causes forced exocytosis of vesicles, and if there were synapses in these regions, they would likely be tracked with the presence of either rhodamine dextran to mark the lumen or FM1-43 to mark the plasma membrane. The drawback of completing this experiment is that it isn't possible to know if these neurons can undergo action potentials in vitro, but it could be helpful in showing whether or not there are synaptic interactions at en passant axons.

References

Harris, TW, Schuske, K, and Jorgensen, EM. 2001. Studies of Synaptic Vesicle Endocytosis in the Nematode *C. elegans, Traffic*. Vol. 2; 597-605.

Barron, B. 2004. Biomedical Science 108 - Human Physiology - Spring 2004. Sited online March 26, 2004 at http://www.biosbcc.net/barron/physiology.

Kandel, ER, Schwartz, JH, and Jessell, TM. 2000. Principles of Neural Science. McGraw-Hill Companies, Inc. United States of America.

Maravall, M, Koh, IK, Lindquist, WB, Svoboda, K. 2004. Experience-dependent Changes in Basal Dendritic Branching of Layer 2/3 Pyramidal Neurons During a Critical Period for Developmental Plasticity in Rat Barrel Cortex. *Cerebral Cortex Advanced Access Online.*

Cooper, Geoffery M., Robert E. Hausman. The Cell: Changes in Molecular Approach. 3rd ed. Washington, D.C.: ASM Press. 2004.

"Dextran Conjugates." Product information. Molecular Probes Website, 2003. Retrieved on March 3, 2004 at http://www2.cbm.uam.es/confocal/pdf%20plasmidos/dextranos.pdf

Bi et al. 1997. Journal of Cell Biology. November 11, 2003. p. 1 RLM class.

Stevens, CF and Williams, JH. 2000. "Kiss and run" exocytosis at hippocampal synapses. *Proceedings of the National Academy of the Sciences*. Vol. 97, no. 23. 12828-12833.

Hollenbeck, PJ, and Morris, RL. 1993. The regulation of bidirectional mitochondrial transport is coordinated with axonal outgrowth. *Journal of Cell Science*. Vol. 104; 917-927.

Savage, AL, Biffin, M, and Martin, BR. (1989). Vasopressin-stimulated Ca2+ influx in rat hepatocytes is inhibited in high-K medium. *Biochemial Journal*. Vol. 260; 821-827.

Special thanks to my collaborator Lauren Suarez for her input and assistance on the protocol, imaging, and entertainment during this experiment. I'd also like to thank Professor Morris with his help on this somewhat 'dependent' research project, and the entire neurobiology class for their help and motivation during those late night hours spent in the Science Center.